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Gene flow in symbionts of *Cladonia arbuscula*

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Abstract: Genetic diversity in symbionts of the lichen *Cladonia arbuscula* was investigated. Forty-eight specimens of *Cladonia arbuscula* located within a 2 km range at Payuk Lake in Manitoba were collected. Population structure and method of symbiont transmission in *C. arbuscula* were inferred by examining the presence or absence of introns in the nuclear small subunit (SSU) ribosomal DNA (rDNA) of the mycobiont and restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) rDNA in the photobiont. The mycobiont showed a high level of genetic variation and the photobiont a low level of variation within the small geographic range studied. An analysis of molecular variance of fungal genotypes showed significant population subdivision for the mycobiont when the data were analysed at $P=0{\cdot}05$. No population subdivision was detected when $P=0{\cdot}001$. The photobiont also showed no population subdivision. Photobiont genotypes did not correlate with mycobiont genotypes suggesting the symbionts were not transmitted together in the same propagule. Multiple fungal genotypes were also detected in *C. arbuscula* and possible explanations were discussed.

Key words: *Cladonia arbuscula*, gene flow, genetic variation, symbiont transmission

Introduction

Genetic variation is generally assumed to be important for a species to adapt to new habitats (Milton & Grant 1984) and is detected in the separate genomes of algal and fungal partners of lichen associations. New patterns of genetic variation can be generated by the occurrence of recombination or maintained by vegetative reproduction. However, the formation of a lichen thallus by two ecologically obligate symbionts, the photobiont and mycobiont, adds another level of variability that may influence survival of the lichen association. Symbiotic variation, the variation between symbiont genotypic patterns, depends on compatibility of the symbionts (Honegger 1996). Since fungal apothecia release ascospores generally without the photobiont, the spores must land and germinate in the vicinity of a compatible photobiont before lichenization can proceed. This presents a challenge for the lichen as two compatible partners must come together before the lichen can develop. The challenge is lessened by production of vegetative propagules of lichens that form packages of fungal hyphae and photobiont cells together in soredia, isidia, or thallus fragments. Since the two partners are already together and compatible in the vegetative propagule, they will develop into another lichen thallus more effectively ensuring that the same symbionts will be found together in the new lichen formed from the fragment. This ensures maintenance of the species but reduces the level of symbiotic and genetic variation.

Genetic variation can be used to infer gene flow. A comparison of genetic variation between symbionts (symbiotic variation) can be used to infer the method of symbiont transmission. Studies of dispersal and gene flow in lichen fungi have included mark-recapture studies where fragments have been marked by colour and monitored in the environment (Heinken 1999), the use of chemical markers (Culberson

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et al. 1988; Culberson *et al.* 1993), or molecular markers based on isozyme studies (Brown *et al.* 1989; Fahselt 1988; Fahselt & Hageman 1983; Fahselt *et al.* 1995; Hageman & Fahselt 1992), microsatellites (Walser *et al.* 2003), RAPDs (Dyer *et al.* 2001), and RFLPs of group I introns in nuclear rDNA (Beard & DePriest 1996; DePriest 1993, 1994). Studies on genetic variation in lichen algae are less common (Kroken & Taylor 2000; Piercey-Normore 2004, 2006; Romeike *et al.* 2002; Tibell 2001; Ohmura *et al.* 2006). Recently, a fungal mitochondrial group I intron has been used to infer population subdivision in *Cladonia subcervicornis* (Vain.) Kernst. (Printzen & Ekman 2003). Dispersal was shown to be limited in *Umbilicaria mammulata* (Ach.) Tuck. (Hageman & Fahselt 1992) and *Lobaria pulmonaria* (L.) Hoffm. (Zoller *et al.* 1999) but thought to be widely effective in *Caliciales* and *Sphaerophoraceae* (Tibell 1994; Wedin 1995). Barriers created by geological formations or vascular plants in the area can also affect the dispersal distance and pattern (Wiens 2001). For example, movement of wind-dispersed propagules may be facilitated by open waterways or hampered by dense forests. Six species of *Cladonia* and one species of *Cetraria* produced negligible dispersal of thallus fragments in a closed old-growth pine forest and maximal dispersal in an open pioneer pine forest (Heinken 1999).

The terricolous reindeer lichen *Cladonia arbuscula* (Wallr.) Flot. is widely distributed throughout northern North America being circumpolar, boreal, and montane (Thomson 1984). It commonly grows over thin soil and on rocky outcrops. The highly branched and brittle thallus of *C. arbuscula* provides abundant fragments for dispersal. There are no specialized symbiotic propagules produced. Pycnidia are common at the branch tips and produce potential vegetative propagules or spermatia for sexual reproduction. If populations reproduce by fragmentation only, then genotypes of fungi and algae should correlate with one another. However, if populations are dispersing by sexual reproduction where the spore must re-lichenize, or through a combination of both sexual and vegetative reproduction, symbiont genotypes would not correlate with one another.

We attempted to reach our goals by using information from the presence or absence of introns in nuclear rDNA in the fungal symbiont. Variation in group I introns has been reported from nucleotide sequences (Myllys *et al.* 2001; Printzen & Ekman 2003) and from the presence or absence of introns (Piercey-Normore 2004; Beard & DePriest 1996), which are common in the SSU of lichen-forming fungi (Gargas *et al.* 1995). Group I introns are thought to code for ribozymes, enzymes that catalyse excision of the intron from the rRNA precursor. Group I introns are usually inserted in the conserved secondary structure of rRNA (reviewed in Haugen *et al.* 2004), and those encoding homing endonuclease genes (HEG) are mobile suggesting that they can be transferred horizontally between different species. Group I introns were initially acquired horizontally at a specific site within some fungal taxa, but after species divergence they were transmitted vertically (Hibbett 1996; Bhattacharya *et al.* 1994, 1996, 2002). Vertical inheritance of some group I intron-like insertions has been suggested in lichen fungi by comparison of phylogenies produced from spacer regions in nuclear ribosomal DNA with those produced from group I introns (Thell 1999; Myllys *et al.* 1999; Thell & Miao 1999; but see also Myllys *et al.* 2001; Simon *et al.* 2005). If these insertions are indeed transmitted vertically they might be useful in population and phylogenetic studies. The presence or absence of these elements has been used to designate strains or populations (Coates *et al.* 2002) and as an indication of genetic variation in population studies (Beard & DePriest 1996).

The objectives of this study were (1) to examine population structure in each of the symbionts in *C. arbuscula* using presence or absence of introns as markers, and (2) to determine the method of symbiont transmission by comparing genotypic variation between symbionts.

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Materials and Methods

Study sites and lichen material

The study sites were located on the Precambrian shield on islands and on the main shore on the eastern side of Payuk Lake, Manitoba (Table 1). One specimen of *Cladonia arbuscula* was collected from each of five contiguous cushions (mats) separated by at least 1 m in each of ten transects providing 50 specimens of lichen thalli (Robertson collection numbers 1–50). All specimens were air dried and deposited in the cryptogamic division of the University of Manitoba Herbarium (WIN). Detection of secondary compounds was performed by thin-layer chromatography (TLC) according to the methods described by Culberson (1972). *Cladonia arbuscula* is often mistaken for *C. mitis* Sandst. especially in Manitoba where the strain of *C. arbuscula* that lacks fumarprotocetraric acid is common. *Cladonia arbuscula* can be recognized by upper branchlets recurved toward one side, and a pale yellow dull surface. The highly branched podetia form large mats and sometimes produce small brown apothecia at the tips of the branches. Two specimens were removed from the analysis since they contained fumarprotocetraric acid in addition to usnic acid.

DNA isolation and amplification

Total DNA was extracted from a 2 cm thallus branch using a CTAB (cetyltrimethylammonium bromide) extraction buffer and a protocol modified from Grube *et al.* (1995). The thallus was examined for discolouration or foreign particles before isolating DNA. DNA was resuspended in sterile dH_2O and amplified by the polymerase chain reaction (PCR). Amplification of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) of the algal partner was from an algal specific primer nr-SSU-1780-5′ Algal (Piercey-Normore & DePriest 2001) and a universal primer ITS4-3′ (White *et al.* 1990). Amplification of three regions of the fungal small subunit (SSU) of the nuclear ribosomal DNA (rDNA) was from different combinations of five primers; 0819-5′ and 1203-3′; 1184-5′ and 1597-3′; and 1410-5′ and 1597-3′ (Gargas & DePriest 1996). The three regions flanked by these primers are presented in Fig. 1. Presence of an intron in region 1 was determined directly from the length of the amplified product using primers 0819-5′ and 1203-3′. Presence of an intron in region 2 was determined from subtraction of amplified product using primers 1184-5′ and 1597-3′ minus 1410-5′ and 1597-3′. Presence of an intron in region 3 was determined directly from the length of the amplified product using primers 1410-5′ and 1597-3′ (Fig. 1).

Amplifications were performed in 20 µl reaction volumes for restriction endonuclease digestions, and in 300 µl volumes for DNA sequencing. Amplifications were performed in 1 × buffer (200 mM Tris-HCl (pH 8·4), 500 mM KCl), with 2·5 units Taq DNA polymerase (GibcoBRL, Burlington, ON, Canada), 200 µmol l^{-1} of each dNTP, 0·5 µmol l^{-1} of primer, and between 10 and 50 ng of DNA. Amplification conditions in a Techne Genius thermal cycler (Fisher Sci, Nepean, ON, Canada) were: initial template denaturing at 94°C for 5 min, then denaturing at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 2 min, for 33 cycles. PCR products were agarose gel purified by crushing frozen blocks of agarose, pipetting the liquid, and precipitating with 0·2 volumes 5M NaCl and 2·5 volumes 100% ethanol. DNA was quantified on 1% agarose gel stained with ethidium bromide.

Restriction endonuclease digestion

Digestions were performed in 20 µl volumes of the manufacturer's buffer for 300 ng of PCR products of 48 samples of *Cladonia arbuscula*. The endonuclease used for the algal ITS rDNA was Hae III (Gibco BRL, Burlington, ON, Canada) and for the fungal introns was Hae III and Hpa II (Gibco BRL, Burlington, ON, Canada). Restriction fragment length polymorphisms (RFLPs) were electrophoresed in a 2% agarose mixture run at 150 volts until the loading dye reached 1cm from the bottom of the gel, stained with ethidium bromide, and visualized by ultraviolet transilluminator. Fragment lengths were determined by comparison with commercial DNA ladders (Gibco BRL, Burlington, ON, Canada).

DNA sequencing and sequence alignment

Double stranded amplicons from two bands in the fungal sample, JR7-4, obtained by amplification with primers 1410-5′ and 1597-3′ were sequenced using BigDye Terminators Version 3.0 on a 377 and 377XL ABI DNA Sequencing Instrument (University Core DNA and Protein Services, University of Calgary, Calgary, Alberta). The DNA sequences were assembled into full-length sequences using Sequencher 4.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Both strands were sequenced from two PCR primers. The ITS rDNA sequences matched known sequences from a BLAST search (Altschul *et al.* 1997) and previous studies (Piercey-Normore 2004; Piercey-Normore *et al.* 2004).

Data analysis

Group I introns in the three regions of fungal rDNA used in this study are part of the same SSU region (Fig. 1). Fungal data were examined as haploid data where the entire SSU region is treated as a single locus with each combination of introns as a single allele. For the analysis multiple alleles were treated as missing data and they were also included in the data. Fungal haploid data were scored as alleles 1 to 11, and 0 represented multiple introns in the same specimen (i.e. missing data). Since the algal ITS rDNA did not contain mobile elements it was analysed as haploid and binary data with two alleles corresponding to two RFLP patterns.

Distribution of fungal SSU genotypes and algal ITS genotypes among transects was examined by Analysis of Molecular Variance (AMOVA) at two levels; within transects (φ_{PR}), and among transects (φ_{PT}), using GenAlEx 6.0 (Peakall & Smouse 2005) with 999 per-

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TABLE 1. *Site description and location of the ten transects used in this study*

Transect number*	Site description	Latitude and longitude
1 Hook Point (Mats 1–5)	Wooded†; on a N-facing slope	101°31′52″N 54°38′15″W
2 Middle Three Islands (Mats 6–9)	Sparsely wooded; on E-facing slope on E side of island	101°32′8″N 54°38′15″W
3 Three Islands Bay (Mats 10–14)	Wooded; on a N-facing slope	101°31′54″N 54°38′15″W
4 My Island South (Mats 15–19)	Wooded; NNW-facing slope up over a ridge to SSE-facing slope	101°31′00″N 54°38′45″W
5 Long Island North (Mats 20–24)	Wooded and exposed; N-facing slope of a ridge is wooded and S-facing slope is exposed	101°31′12″N 54°38′36″W
6 Long Island South (Mats 25–29)	Exposed; SE-facing slope	101°31′45″N 54°38′30″W
7 Red Rock Island (Mats 30–34)	Wooded; flat	101°31′6″N 54°38′24″W
8 Red Rock Creek (Mats 35–39)	Wooded with large boulders; N-facing slope	101°30′48″N 54°38′24″W
9 My Island North Point (Mats 40–44)	Sparsely wooded; WNW-facing slope over a bluff	101°30′30″N 54°38′48″W
10 Mystic Channel West Island (Mats 45–50)	Sparsely wooded and exposed; SE-facing slope along a ridge crest	101°31′18″N 54°38′54″W

*numbers correspond to those in Fig. 5.
†wooded areas contain predominantly white spruce and jack pine.

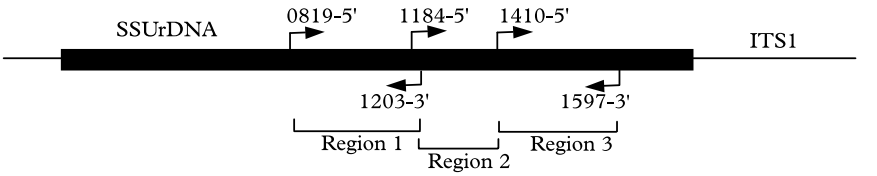

FIG. 1. Location and direction of primers in the fungal SSU rDNA producing three amplification products (SSU regions 1, 2, and 3). Presence of an intron in region 1 was determined directly from the length of the amplified product using primers 0819-5′ and 1203-3′. Presence of an intron in region 2 was determined by the length of amplified product between primers 1410-5′ and 1597-3′ subtracted from length of amplified product between primers 1184-5′ and 1597-3′. Presence of an intron in region 3 was determined directly from the length of the amplified product using primers 1410-5′ and 1597-3′.

mutations. φ_{PT} (Peakall & Smouse, 2005) is a measure of population differentiation that is analogous to F_{ST} (Weir & Cockerham 1984) where φ_{PT} is calculated as the proportion of variance among populations (V_{AP}) relative to the total variance ($V_{AP}+V_{WP}$). V_{WP} is the variance within populations. Therefore, $\varphi_{PT}=V_{AP}/(V_{AP}+V_{WP})$. The term "population" refers to transect in this study.

The statistical package, SPSS ver. 8.0 (SPSS Inc., Chicago, Illinois), was used to test for Spearmans correlation coefficient. Correlations were tested between algal and fungal genotypes, fungal genotype and the presence of apothecia, and between algal genotype and presence of fungal apothecia to determine the method of fungal and algal transmission and reproductive modes. The minimum spanning tree was produced from HIERCLUS in SYN-TAX 5.02 (Podani 1994) using Euclidian distances of binary data excluding the samples that contained multiple bands. The stippled areas in Fig. 5 enclose all transects containing common genotypes (found more than 5 times) shared among sites.

Results

Genetic variation in the mycobiont

Fungal genetic variation was determined from the presence or absence of introns in each of three amplified regions in the nuclear SSU rDNA (Fig. 1) between positions 819 to 1597 (Gargas & DePriest 1996). The length of the total amplified coding region of the SSU rDNA between these positions was predicted to be 778 bp. Amplified product that was 250, 500, or 750 bp longer than the expected SSU coding region represented group I intron-like insertions. The presence or absence of introns in each of the three SSU regions was scored as haploid data. For haploid data there were 11 different patterns of intron presence (genotypes) (Fig. 2) and 8

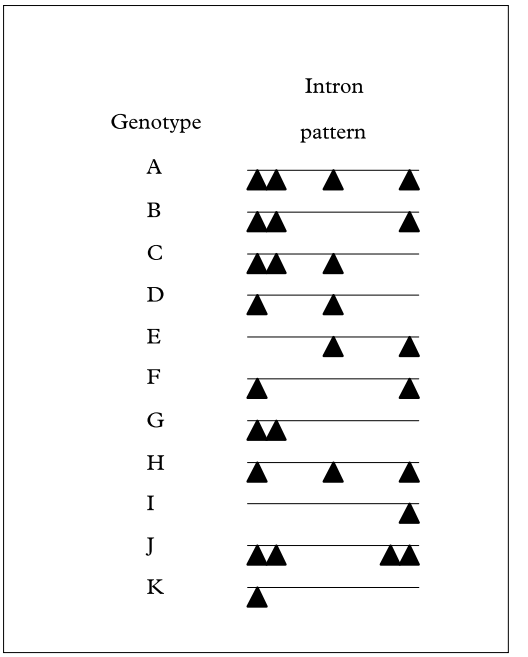

FIG. 2. Fungal SSU haploid genotypes present in 48 samples of *Cladonia arbuscula* based on the presence or absence of introns in three amplified regions of SSU rDNA producing 11 different genotypes. The horizontal line represents the SSU and the solid triangles indicate the presence of an intron. Although two triangles in position 1 indicate two introns, the position of a single intron cannot be determined.

(of 50) samples showing multiple genotypes in the same individual. From 11 haploid genotypes there were five unique and six common genotypes. A comparison of fungal genotypes across all transects showed that some genotypes were more common than others (Table 2). Genotypes A, B, C, D, F,

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TABLE 2. *Proportions of fungal genotypes in* Cladonia arbuscula *from ten transects. Genotypes were determined from haploid data of the SSU rDNA amplified region. Multiple alleles were excluded*

	Transect									
Fungal genotype	1	2	3	4	5	6	7	8	9	10
A	0·20	0·00	0·50	0·20	0·40	0·00	1·00	0·00	0·00	0·00
B	0·20	0·00	0·00	0·00	0·00	0·00	0·00	0·25	0·20	0·00
C	0·20	0·33	0·00	0·20	0·20	0·00	0·00	0·00	0·60	0·00
D	0·20	0·00	0·00	0·40	0·00	0·25	0·00	0·25	0·00	1·00
E	0·20	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
F	0·00	0·33	0·00	0·20	0·00	0·25	0·00	0·25	0·00	0·00
G	0·00	0·33	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
H	0·00	0·00	0·50	0·00	0·40	0·00	0·00	0·25	0·20	0·00
I	0·00	0·00	0·00	0·00	0·00	0·25	0·00	0·00	0·00	0·00
J	0·00	0·00	0·00	0·00	0·00	0·25	0·00	0·00	0·00	0·00

and H occurred three or more times. Genotypes E, G, I, and J occurred only once and are unique genotypes. Genotype K was present only within a multiple allele. The most common genotype, genotype A, contained all four introns, and was present in nine specimens. To determine genotypes the absence of an intron in region 1 was identified by amplified product 385 bp long, the presence of an intron was 635 bp, and the presence of 2 introns was 885 bp. The position of these inferred introns in region 1 cannot be distinguished if a single intron is found. The absence of an intron in region 2 was identified by amplified product 225 bp long, and the presence of an intron was 505 bp. The absence of an intron in region 3 was identified by amplified product 190 bp, and the presence of an intron was 440 bp (Fig. 3A). The presence of an intron in the same SSU region in all five specimens from a single transect produced a monomorphic band and suggested no polymorphism. Therefore, these five specimens were subjected to RFLPs to determine sequence variation. In the five specimens in transect 7 there was more than one different RFLP pattern detected suggesting variation in intron DNA sequence (Fig. 3B). Genotype frequencies showed that the presence of introns was more common than the absence of introns in the SSU rDNA. The presence of two introns was common in region 1 but rare in region 3 where they occurred only once.

Genetic variation in the photobiont

Algal genetic variation was determined from RFLP of the ITS nuclear rDNA sequence. The uncut ITS region was 560 bp long. RFLP produced two patterns representing two genotypes (Fig. 3C). Genotype I (single band at 200 bp) was present in 81% of the samples and genotype II (two bands at 200 bp and 190 bp) was present in 15% of the samples. Two samples did not amplify. There were other unresolved fragments shorter than 100 bp that were not used to define the two genotypes. The photobiont showed no population subdivision in either haploid (φ_{PT}=0·043) or binary (φ_{PT}=0·043) data analyses.

Mycobiont population structure

Significant population subdivision at P=0·05 was detected for haploid fungal data when multiple alleles were treated as missing data (φ_{PT}=0·098) suggesting that gene flow was limited among transects. However, there was no population subdivision at P=0·001 or when multiple alleles were included in the data (φ_{PT}=0·019). Pairwise comparison between transects indicated gene flow between most transects (Table 3).

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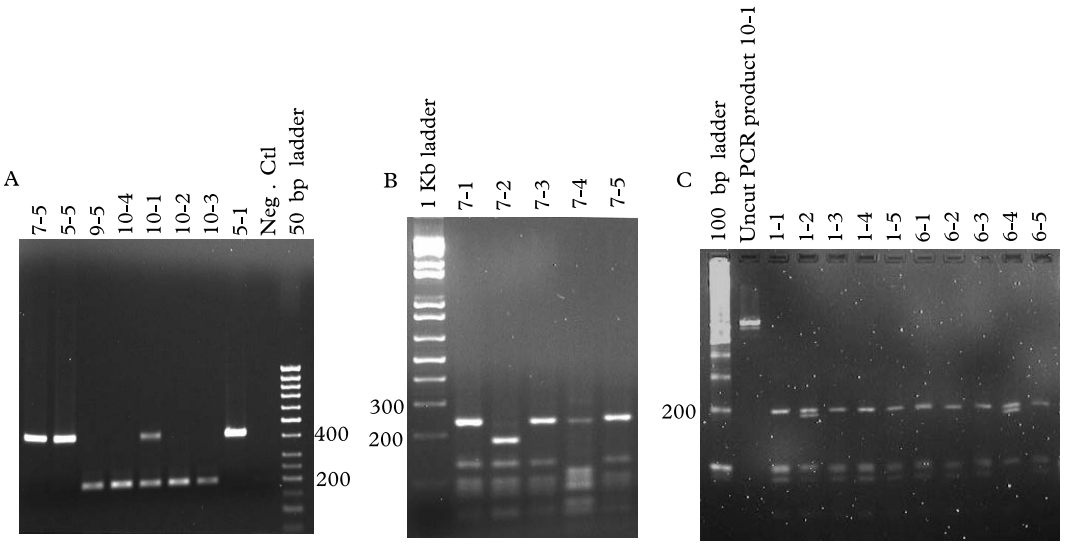

FIG. 3. A, banding pattern produced by PCR amplification with fungal primers 1410-5′ and 1597-3′ (region 3) showing the allele amplified when an intron was absent from the SSU region (200 bp), when an intron was present (450 bp), and when both alleles (intron absent and present) were found in the same sample (lane 10-1); B, RFLP of a monomorphic band from SSU region 3 containing an intron in all five samples, the five samples were collected from transect 7, after digestion with a restriction enzyme lane 7-2 produced a banding pattern different from the other lanes, lane 7-4 contained multiple genotypes; C, RFLP of the algal ITS rDNA showing genotype I with a single 200 bp band and genotype II with a band at 200 bp and another band at 190 bp. All lanes are labelled with transect and sample numbers.

TABLE 3. *Pairwise φ_{PT} values among ten transects of the fungal SSU rDNA haploid data set in* Cladonia arbuscula

Transect	1	2	3	4	5	6	7	8	9
2	0								
3	0·115	0·010							
4	0	0	0·167						
5	0	0·059	0·107	0·034					
6	0	0	0·034	0	0·100				
7	0·167	0·289*	0·236	0·226	0·118	0·271*			
8	0	0	0	0	0·022	0	0·271		
9	0	0·010	0·271*	0·091	0·063	0·150	0·450*	0·076	
10	0·098	0·200	0·285	0	0·337*	0·043	0·532*	0·043	0·391*

*significant at P=0·05; none of the values are significant at P=0·001.

Significant population subdivision occurred between transect 7 and transects 2 and 6, and between transect 9 and transects 3 and 7, and between transect 10 and transects 5, 7 and 9 (Table 3) suggesting low gene flow between these transects. Pairwise comparisons between all other transects (Table 3) suggested more gene flow.

The occurrence of multiple fungal alleles was indicated by the presence of two bands of different lengths in the same sample (Fig. 3A, lane 10-1) suggesting that two alleles

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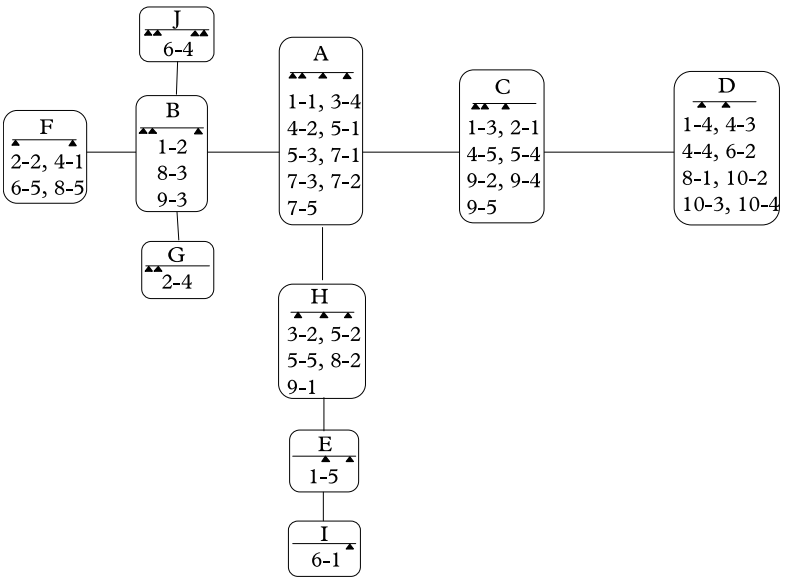

FIG. 4. Minimum spanning tree showing clustering of transects and mats of *C. arbuscula* based on fungal genotypes defined by presence or absence of group I introns in three adjacent regions of the fungal SSU rDNA (see Fig. 1). Genotypes are represented by letters (see Fig. 2), transects and mats are indicated by the first number as the transect, and the second number after the hyphen as the mat within the transect. Genotype K was not included in the analysis because it was present only within a multiple allele and not detected as a separate sample.

were present in the sample, i.e. intron present and intron absent. The presence of multiple alleles was more common in SSU region 3 than in the other two SSU regions. In total there were eight samples with multiple alleles. This is probably a conservative number since multiple genotypes consisting of different intron sequences of the same length would not be detected using this method. The DNA sequence of one of these samples containing two bands (JR7-4) revealed that the short fragment was the SSU coding sequence and the long fragment was the same SSU coding sequence with an intron inserted at position 1199 (DQ238108). Since all other samples with double bands were approximately 250 bp apart, they were assumed to be one band without an intron and one containing an intron. A BLAST search was performed on the JR7-4 sequence and produced significant similarity with other introns at position 1199 in *Gymnoderma coccocarpum* Nyl. and *Cladonia gracilis* (L.) Willd.

Movement of propagules

There was no significant correlation between fungal genotype and algal genotype ($r = +0{\cdot}019$, $P = 0{\cdot}345$); between fungal genotype and presence of apothecia ($r = +0{\cdot}076$, $P = 0{\cdot}304$); or between algal genotype and presence of fungal apothecia ($r = -0{\cdot}052$, $P = 0{\cdot}366$). Apothecia were present in 40% of the samples.

A minimum spanning tree clustered identical genotypes together showing genotype A, a genotype with the largest number of introns, as the genotype in the centre of the tree (Fig. 4). The tree shows how loss of introns could produce the genotypes detected in this study. The mapping of the four most common genotypes (A, C, D, and H) on a geographic map, based on the minimum spanning tree, produced two patterns; genotypes located in the north and south clustered together (Fig. 5C), genotypes located between islands and the main shore (Fig. 5A & B, D) clustered together.

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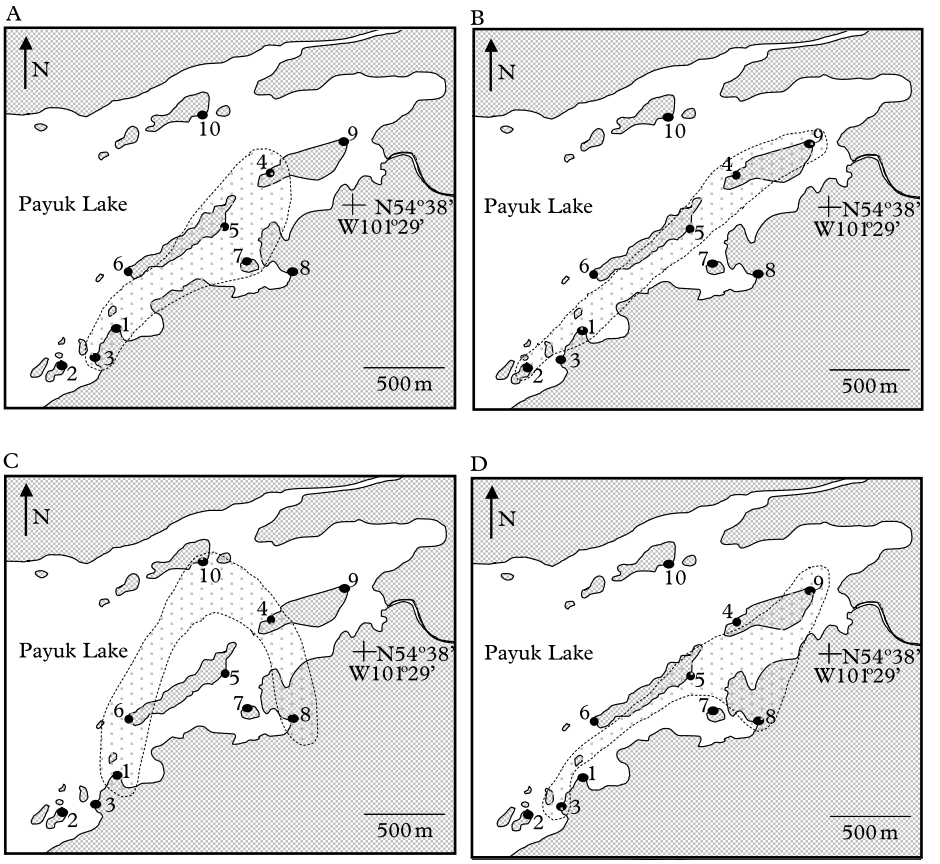

FIG. 5. Map of the study site at Payuk lake, Manitoba, Canada, showing location of 10 transects (numbered 1–10). Shaded area represents land mass and clear area represents water. Stippled areas that encircle several transects represent those genotypes that were clustered in Fig. 4. A, transect cluster based on the presence of genotype A; B, transect cluster based on the presence of genotype C; C, transect cluster based on the presence of genotype D; D, transect cluster based on the presence of genotype H.

Discussion

Level of genetic variation in the mycobiont

A higher degree of fungal variation in a small geographic area was evident in this study compared with other studies. Although Printzen & Ekman (2003) reported fungal diversity higher than that in other studies, they reported only seven different haplotypes in *C. subcervicornis*, in a 45 km range. Beard & DePriest (1996) reported three different genotypes in *C. subtenuis* (Abbayes) Mattick in *c.* 1000 km range. Lindblom & Ekman (2006) reported 10 intergenic spacer (IGS) and 16 ITS haplotypes in *Xanthoria parietina* in a 3 km range. By contrast this study produced eleven genotypes in a 2 km range. The type of marker (presence/absence of introns) used in this study was similar to that used in Beard & DePriest (1996; introns and RFLP) but different from Printzen & Ekman (2003; nucleotide sequence of COX1 intron). DNA sequence data from spliceosomal introns in large subunit rDNA produced only six genotypes in *Lobaria pulmonaria* in *c.* 200 km range (Zoller *et al.* 1999), and the ITS

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rDNA produced only four fungal genotypes in *Parmotrema tinctorum* (Delise ex Nyl.) Hale in 60 km^2 (Ohmura *et al.* 2006). However, variation in simple sequence repeat microsatellite regions in *L. pulmonaria* was higher. Walser *et al.* (2003) revealed 27 haplotypes in four loci from 30 samples collected from different continents. Comparison of polymorphism is difficult because of variability in geographic ranges and the use of different types of markers. The high level of variation reported in the small geographic range in this study could be explained by horizontally mobile introns or a rapid loss of introns in some populations but not in others. The presence of five unique genotypes in this study suggested that a larger geographic scale and sample size may produce a larger amount of genetic variation using the presence or absence of group I introns as markers. The number of haploid alleles ranged from one to a maximum of five in each transect. That five fungal genotypes were detected in five samples suggested this sample size was not large enough to detect the actual diversity present in the area.

Transmission of algal and fungal symbionts

Comparison of algal and fungal genotypes did not produce a significant correlation suggesting that the symbionts were not distributed together in thallus fragments. This was also reported between symbionts of *Parmotrema tinctorum* (Ohmura *et al.* 2006). Several explanations may provide insight into the method of dispersal for each symbiont. (1) Dispersal may be from independent ascospores or conidia where the spore must re-lichenize with a potentially different algal partner at each dispersal event. The presence of apothecia (40%) would support this hypothesis but little is known about the viability of fungal spores in *Cladonia*. (2) Dispersal may be from fragments with exchange of algal partners after the fragment falls onto a lichen thallus in a suitable habitat. The fungus in the fragment may incorporate the alga from the thallus (Friedl 1987; Ott 1987; Ott *et al.* 1995) or it may associate with a free-living alga in the vicinity (Bubrick *et al.* 1984). (3) On the other hand, the assumption was made that the fungal SSU genotypes reflect the fungal genomic variation. If the group I introns in the fungal SSU are independent and functionally mobile elements they would not reflect fungal genomic variation.

Algal genotype I was predominant in the study even though the abundance of apothecia provided opportunity for the fungus to re-lichenize with a different algal partner. Since 15% of the lichen thalli contained algal genotype II, both genotypes were available for association with *C. arbuscula*. The predominance of algal genotype I (81%) may be explained by a number of factors with influence from environmental conditions and fungal genetics. In another study genotype II was shown to be present in the geographic area in association with *C. gracilis*, but it was absent in *C. rangiferina* (L.) F. H. Wigg. and *C. multiformis* G. Merr. (Piercey-Normore 2004). The distribution of algal genotypes among species or populations of lichen-forming fungi is unknown, but an absence of phylogenetic congruency has been shown at the population (Yahr *et al.* 2004) and higher taxonomic (Piercey-Normore & DePriest 2001) levels.

Gene flow in *Cladonia arbuscula*

Significant population subdivision was present among transects (at $P=0{\cdot}05$), but only when multiple alleles were treated as missing data. The significance in the data set may be influenced by several factors. (1) A small data set and sample size with a large number of alleles (haploid data set) may produce a large variance influencing the calculation of φ_{PT}. (2) Although evidence has been presented to support vertical transmission and negligible horizontal movement of group I introns (Hibbett 1996; Bhattacharya *et al.* 1994, 1996, 2002), introns have been hypothesized to be mobile in the large subunit of nuclear ribosomal RNA in the *Physciaceae* (Simon *et al.* 2005) and this was suggested to be a possible explanation for the incongruency between

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rDNA and intron trees in *Physcia* (Myllys *et al.* 2001). If these introns are mobile then the same population could be represented by more than one haploid genotype, inflating the amount of variation actually present. The presence of non-homologous introns may also influence the amount and type of variation. Non-homology of presence/absence data has been a problem with other methods such as RAPDs (Randomly Amplified Polymorphic DNA). A parallel study using a coding or intronless gene would shed light on congruency of the patterns of variation between introns and vertically transmitted coding genes (e.g. Thell 1999; Myllys *et al.* 1999; Thell & Miao 1999). Printzen & Ekman (2003) used the group I intron in the mitochondrial cytochrome oxidase 1 as a population marker despite their finding that it contained an open reading frame for a homing endonuclease gene providing evidence for potential horizontal movement.

Population subdivision was reported between seven pairs of transects at $P=0{\cdot}05$ and between no transects at $P=0{\cdot}001$ (Table 3) suggesting that gene flow occurred among transects. Gene flow may be influenced by recent population expansion, isolation of populations, unsuitable habitat, clonal propagation, and production of insufficient numbers of propagules. Recent population expansion cannot be eliminated because the area sampled was so small and probably included the entire biological population. Isolation of populations may result from large distances between transects, islands forming a barrier to wind and water flow, or the dense forest around transect 7 (Table 1) providing protection against wind. Heinken (1999) demonstrated lower dispersal by fragmentation in a closed forest than in an open forest. If highly branched *Cladonia* species propagate clonally and disperse short distances, then low variation would be expected (Beard & DePriest 1996). Suitable habitat, thin soil over rock outcrops, is widespread in this area of Manitoba and would account for the common occurrence of *C. arbuscula*. Sufficient numbers of propagules are evident from the large numbers of fragments produced when the brittle thallus is crushed, and it is assumed from the abundance of apothecia observed on the podetia. However, little is known about dispersal vectors for thallus fragments and viability of ascopores in *Cladonia*.

Gene flow between transects is suggested by lower φ_{PT} values in most pairwise comparisons (Table 3). Transects containing common genotypes were clustered together (Fig. 4) and mapped onto a geographic map (Fig. 5). The stippled areas suggest two patterns. One pattern is between the islands and the main land mass (Fig. 5A, B and D). The other pattern is between opposite sides of the lake (Fig. 5C). Samples from transect 10 were located on an exposed ridge (Table 1). Perhaps winter storms carried lichen fragments across the frozen lake to the islands and the southern shore. Similarly, genotypes between transects 1 to 9 may have been carried by wind or animals travelling between the islands and the main shore over ice in the winter. The geographic distance in this study was smaller than that in Printzen & Ekman (2003) for *C. subcervicornis* where long distance dispersal was considered to be ineffective, but dispersal was detected among three sites approximately 10–15 km apart. Gene flow was also detected in *Xanthoria parietina* at distances of one or a few km (Lindblom & Ekman 2006). On the other hand, the common genotypes may have arisen independently, suggesting that no gene flow has occurred. This explanation is plausible because of the nature of mobile elements but contradicts the results of the AMOVA analysis.

Multiple fungal genotypes

Since most ascomycetes are thought to be monokaryotic and haploid (Alexopoulos *et al.* 1996) a single band should be amplified from a single SSU region. However, two alleles, one containing an intron and the other without an intron, were amplified from 10 SSU regions (in 8 specimens) in this study (Fig. 3A, lane 10-1). The presence of multiple fungal genotypes was also reported by Murtagh *et al.* (2000) and may be explained by at least five possible scenarios.

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(1) The dikaryotic and diploid phases restricted to the ascogonium and formation of the apothecium contain two sets of chromosomes, one with and one without an intron. However, the dikaryon and diploid phases in the DNA from a single podetium would make up such a small proportion of isolated DNA that it is unlikely to be amplified to a high degree as in this study. (2) Although uncommon in ascomycetes (Alexopoulos *et al*. 1996) the sample may represent an extended dikaryotic phase forming the thallus. (3) The podetium used for DNA extraction may consist of at least two separate fungal individuals or mating types. If this species of *Cladonia* is heterothallic as is reported for *C. floerkeana* (Fr.) Flörke, *C. galindezii* Øvstedal, and *C. portentosa* (Dufour) Coem. (Seymour *et al*. 2005) both mating types would be required for sexual reproduction and formation of apothecia (40% of the samples). This might suggest that both mating types (and possibly two genotypes represented by presence and absence of an intron) be present in the same podetium. Although DePriest (1993) reported chemistry of two separate species in a single individual of *C. chlorophaea* (Flörke ex Sommerf.) Spreng. complex, she maintained that it was not evidence for hyphal mixing. Nevertheless Jahns (1988) suggested that podetia of *Cladonia* frequently represent mixtures of hyphae of different genotypes. (4) The intron may not be fixed in all repeats of the tandemly repeated rDNA indicating a failure of concerted evolution. On the other hand, incomplete concerted evolution of a currently mobile intron would also produce intron-present and intron-absent alleles in the same individual (Simon *et al.* 2005). Failure of concerted evolution was suggested to be the cause of a non-uniform evolution between the external transcribed spacer and the ITS of an individual genome in *Mitella* (Saxifragaceae) (Okuyama *et al*. 2005). (5) Paralogous genes would also explain the presence of two alleles. Paralogy could be detected by examination of the DNA sequence for numerous substitutions in the coding regions of highly conserved SSU flanking the introns (Razafimandimbison *et al.* 2004).

In conclusion, this study reported a high level of variation in *Cladonia arbuscula* within a small geographic area and provided some evidence for population subdivision using haploid data. However, greater evidence for lack of population subdivision suggested gene flow between populations of *C. arbuscula* for both symbionts. The method of gene flow was not solely by fragmentation but may have been a combination of fragmentation, ascospores, conidia and horizontal transfer of algal partners between fungi. The presence of multiple alleles within a thallus raises interesting questions about the definition of a fungal ''individual''.

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